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MicroReview

Specificity and selectivity determinants of peptide transport in *Lactococcus lactis* and other microorganisms

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Summary

Peptide transport in microorganisms is important for nutrition of the cell and various signalling processes including regulation of gene expression, sporulation, chemotaxis, competence and virulence development. Peptide transport is mediated via different combinations of ion-linked and ATP-binding cassette (ABC) transporters, the latter utilizing single or multiple peptide-binding proteins with overlapping specificities. The paradigm for research on peptide transport is *Lactococcus lactis*, in which the uptake of peptides containing essential amino acids is vital for growth on milk proteins. Differential expression and characteristics of peptide-binding proteins in several *Lactococcus lactis* strains resulted in apparent conflicts with older literature. Recent developments and new data now make the pieces of the puzzle fall back into place again and confirm the view that the oligopeptide-binding proteins determine the uptake selectivity of their cognate ABC transporters. Besides reviewing the current data on binding specificity and transport selectivity of peptide transporters in *L. lactis*, the possible implications for peptide utilization by other bacterial species are discussed.

Introduction

Lactic acid bacteria (LAB) contain a proteolytic system that enables them to grow in milk. The milk proteins, α-, κ-, and β-casein, are degraded by the cell wall-associated proteinase PrtP and the resulting peptides, of which the transport of oligopeptides is most important for nutrition (Kunji *et al*., 1998), are taken up by the cell. Oligopeptides are defined here as peptides of 4–35 residues. Internalized peptides are further hydrolysed by a large array of peptidases (Kunji *et al*., 1996). Regulation of peptide transport is accomplished via the pleiotropic transcriptional repressor CodY, which senses the intracellular pool of branched-chain amino acids (Guédon *et al*., 2001b).

Lactococcus lactis MG1363 is a plasmid-free derivative of strain NCDO712 and the best characterized LAB in terms of proteolysis, peptide transport and regulation thereof (Tynkkynen *et al*., 1993; Hagting *et al*., 1994; Foucaud *et al*., 1995; Kunji *et al*., 1995; 1996; 1998; Detmers *et al*., 1998; Guédon *et al*., 2001a,b; Kok and Buist, 2003; Sanz *et al*., 2003; 2004). The genome sequence of *L. lactis* MG1363 will be published in the coming year. The chromosome of *L. lactis* IL1403 was the first lactococcal genome to be sequenced (Bolotin *et al*., 2001), and the genomic data from this strain have been combined with experimental data of MG1363. However, the IL1403 and MG1363 genome sequences are on average only ~85% identical, and the percentage identities vary strongly when individual genes are compared (unpubl. result). In recent years, several *L. lactis* strains other than MG1363 have been used in studies of (oligo)peptide transport (Charbonnel *et al*., 2003; Lamarque *et al*., 2004). Apparent discrepancies have arisen in the literature because of (i) the assumption that there is only one oligopeptide transporter (Opp) that is solely responsible for the uptake of oligopeptides in all lactococcal strains, (ii) the erroneous annotation of the Dpp system of IL1403 as Opt (the *dppAPBCDF* and *optSABCDF* gene products are on average 97% identical at the amino acid level), and (iii) differential expression of (oligo)peptide transporters in the studied *L. lactis* strains.

Although at the moment not recognized as such, recent work (Sanz *et al*., 2003; Lamarque *et al*., 2004; this work) provides an explanation for apparent discrepancies in

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published papers (Charbonnel *et al*., 2003; Helinck *et al*., 2003; Doeven *et al*., 2004). In this paper, we present an overview of peptide transport and regulation in *L. lactis* species, and provide alternative explanations for published works. In addition, data are presented that support our current view that the oligopeptide-binding proteins determine the uptake selectivity of the oligopeptide ATP-binding cassette (ABC) transporters.

Peptide transport in *Lactococcus lactis* MG1363

Biochemical analysis indicated that *L. lactis* MG1363 has at least three functional peptide transport systems (Fig. 1). The *opp* and *dtpT* genes were cloned and characterized first and the corresponding transport proteins (Opp and DtpT) were shown to catalyse the uptake of oligo- and di/tripeptides respectively (Tynkkynen *et al*., 1993; Hagting *et al*., 1994). The Opp system belongs to the ABC transporter superfamily and is composed of five proteins: an oligopeptide-binding protein (OppA), two integral membrane proteins (OppB and OppC) and two nucleotide-binding proteins (OppD and OppF). DtpT is a secondary transporter belonging to the PTR family of peptide transporters. An *opp* knockout strain was completely blocked in the uptake of β-casein-derived oligopeptides, indicating that the Opp system is essential for the organism to grow on milk (Tynkkynen *et al*., 1993).

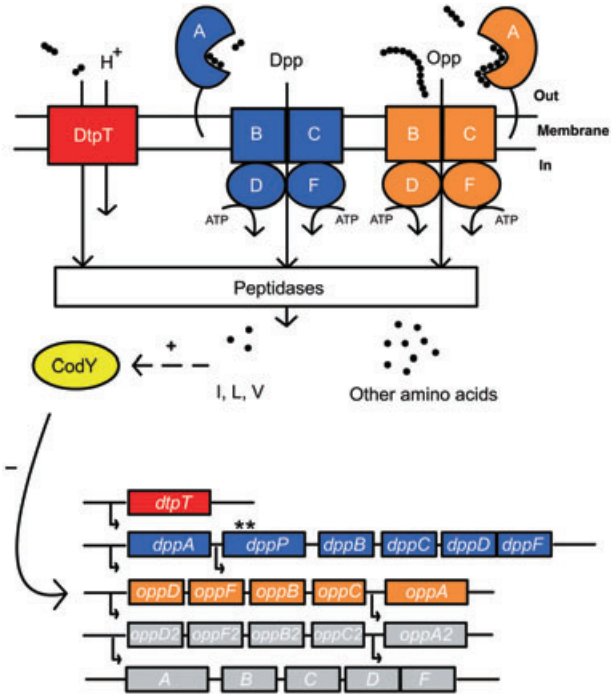

Fig. 1. Schematic overview of function, regulation and genetic organization of peptide transporters in *Lactococcus lactis* MG1363. Three functional peptide transport systems have been identified in *L. lactis* MG1363. The ion-linked transporter DtpT and the ABC transporter Dpp facilitate the uptake of di- and tripeptides. A second ABC transporter, Opp, catalyses the uptake of oligopeptides containing 4 up to and including 35 amino acid residues. The (oligo)peptide-binding proteins (DppA and OppA) are anchored to the membrane via lipid modification of the N-terminal cysteine residue and deliver peptides to their cognate membrane complexes. Internalized peptides are hydrolysed by intracellular peptidases. The transcriptional repressor CodY senses the internal pool of branched-chain amino acids (I, L and V), and inhibits the transcription of, among others, the *opp* genes. In MG1363, the gene coding for the second peptide-binding protein of the Dpp system, *dppP*, contains nonsense and frameshift mutations, which are indicated by asterisks. Recently, a second set of oligopeptide transporter genes, designated *oppD2F2B2C2A2*, and a novel dipeptide/oligopeptide/nickel transporter homologue (genetic organization *ABCDF*) have been found. The Opp2 system and the novel dipeptide/oligopeptide/nickel transporter homologue are either not functional in peptide transport or not expressed.

Analysis of *opp* and/or *dtpT* single or double knockout mutants revealed the presence of a third peptide transport system with preference for hydrophobic (branched-chain amino acid-containing) di- and tripeptides (Foucaud *et al*., 1995). Peptide transport via this third system was dependent on ATP, or a related energy-rich phosphorylated intermediate, and the system was initially designated DtpP. The genes belonging to the DtpP system were cloned, characterized and named Dpp (Sanz *et al*., 2001; Fig. 1). The Dpp system, like Opp, is an ABC transporter. In MG1363, six *dpp* genes are present coding for two peptide-binding proteins (DppA and DppP), two integral membrane proteins (DppB and DppC) and two nucleotide-binding proteins (DppD and DppF). In *L. lactis* MG1363, the *dppP* gene is not functional because of a nonsense mutation and a frameshift (Sanz *et al*., 2001).

Recently, a second set of oligopeptide transporter genes was found in *L. lactis* MG1363, presumably originating from a duplication of the *opp* operon, and the genes were designated *oppD2F2B2C2A2* (Sanz *et al*., 2004; 2005; Fig. 1). An *oppA* deletion mutant was fully impaired in oligopeptide utilization, indicating that in MG1363 this second Opp system is either not expressed or not functional. Interestingly, complementation of an *oppA* knockout with the putative peptide-binding protein OppA2, expressed from a plasmid, restored the ability of the strain to utilize oligopeptides. This indicates that the OppA2 protein can interact functionally with the OppBCDF translocator. Genome analysis of *L. lactis* MG1363 revealed that, next to *opp* and *opp2*, MG1363 contains another dipeptide/oligopeptide/nickel transporter homologue (Fig. 1). At present, it is not clear if this system is expressed and whether or not it is functional as a peptide transporter. On the basis of the oligopeptide null phenotype of the Δ*opp* strains, it is clear that this system does not play a significant, if any, role in oligopeptide uptake.

Regulation of peptide transport in MG1363

Transcription of the genes coding for the proteinase (*prtP*), the oligopeptide transporter (*oppDFBCA*) and several

peptidases (*pepC*, *pepN* and *pepO*) is repressed five to 150-fold upon the addition of peptides to the growth medium, which indicates a negative feedback mechanism of regulation (Detmers *et al*., 1998; Guédon *et al*., 2001a). A systematic study, using 67 dipeptides, showed that regulation of peptide transport was accomplished via a pleiotropic transcriptional repressor, CodY, which senses the internal branched-chain amino acid pool (Guédon *et al*., 2001b). In accordance, deletion of the *dtpT* gene resulted in an upregulation of Opp activity (Kunji *et al*., 1995), suggesting that di/tripeptide transport indeed plays an important role in the regulation of the proteolytic system. Moreover, the expression of Opp was optimal in chemically defined medium (CDM) containing free amino acids, presumably because these are less efficiently internalized than peptides. *In vitro* experiments confirmed that CodY binds upstream of its target genes, i.e. *pepN*, *pepC*, *oppepO1*, *prtPM* and probably *pepX*, and *pepDA2* (Den Hengst *et al*., 2005). Binding of CodY to the promoter of the *opp* system is strongly affected by the addition of branched-chain amino acids. DNA binding is not influenced by GTP, contrary to what was shown for CodY from *Bacillus subtilis* (Shivers and Sonenshein, 2004). A schematic overview of the different peptide transporter genes, their corresponding transport systems, and the regulation of peptide transport in *L. lactis* MG1363 is presented in Fig. 1.

Peptide transport in *Lactococcus lactis* strains other than MG1363

Homologues of all the peptide transporters that are functional in *L. lactis* MG1363 (DtpT, Dpp and Opp) have been found in the sequenced strain IL1403 (Bolotin *et al*., 2001; Fig. 2A). Interestingly, the gene coding for the second peptide-binding protein of the Dpp system of IL1403, *dppP*, does not contain the nonsense and frameshift mutations present in *dppP* from MG1363 (Bolotin *et al*., 2001; Sanz *et al*., 2001). Peptide binding experiments showed that DppP bound di-, tri- and tetrapeptides, with highest affinity for tripeptides (Sanz *et al*., 2003). Peptides longer than four residues were not tested in this study. Importantly, *in vivo* analysis of Dpp in *L. lactis* IL1403 suggests that DppP allows the Dpp system to transport peptides containing up to and including nine amino acid residues (Lamarque *et al*., 2004). Moreover, Western blotting confirmed that DppP is not produced in MG1363, whereas IL1403 does not appear to synthesize the oligopeptide-binding protein (OppA) of the Opp system. This suggests that the critical activity of oligopeptide uptake in IL1403 is not specified by Opp but rather by Dpp (Fig. 2B). Three other *L. lactis* strains (SK11, SKM6 and Wg2) were also tested for their capacity to express (oligo)peptide transport systems. SK11 and Wg2 produced both DppP and OppA, whereas SKM6, like IL1403, only synthesized DppP (Fig. 2B and C). Thus, *L. lactis* SK11 and Wg2 are equipped with at least two functional oligopeptide transport systems. It is not clear whether IL1403 fails to produce only OppA or also its cognate membrane complex OppBCDF. If OppBCDF is expressed, DppP might function not only with the DppBCDF complex but also with OppBCDF, thereby providing the cell with a higher capacity to transport oligopeptides. An overview of the genetic organization and the types of (oligo)peptide transporters in *L. lactis* IL1403 and other lactococcal strains is presented in Fig. 2.

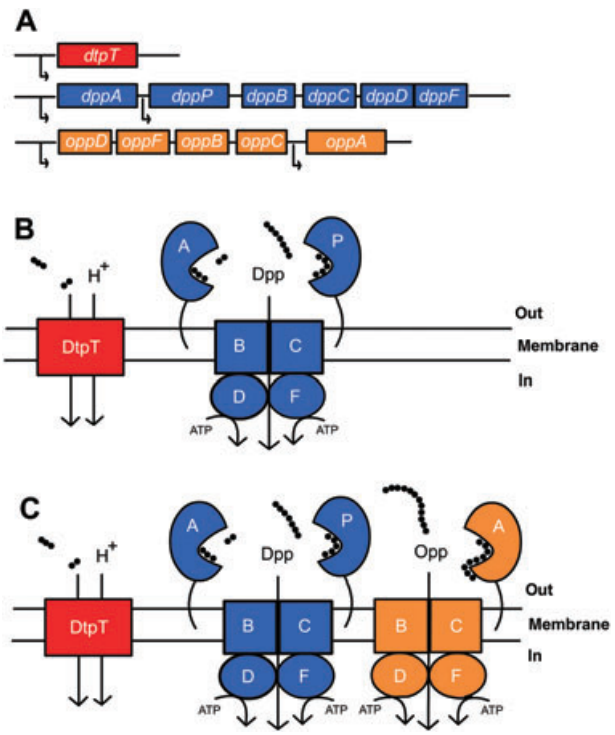

Fig. 2. Schematic overview of genetic organization of peptide transporter genes and function in *Lactococcus lactis* IL1403 and other lactococcal strains. DtpT is presumed to be functional in all lactococcal strains.
A. Genetic organization of peptide transporter genes in *L. lactis* IL1403. The *dppP* gene does not contain the nonsense and frameshift mutations observed in MG1363.
B. In IL403 and SKM6 the Dpp system is used for the uptake of di/tripeptides (DppA) and oligopeptides (using DppP). The Opp system is not used due to lack of OppA production.
C. *L. lactis* strains SK11 and Wg2 are equipped with at least two functional oligopeptide transporters, Opp and Dpp; the latter employing DppP as an oligopeptide-binding protein.

In vivo or *in vitro* studies of peptide transport?

The specificity of peptide transport via the different uptake systems is often evaluated in growth experiments using either wild-type or transport-null strains (Tynkkynen *et al*., 1993; Foucaud *et al*., 1995; Sanz *et al*., 2001; Charbonnel

et al., 2003). As *L. lactis* is auxotrophic for several amino acids such as Glu (Gln), Met, His, Leu, Ile and Val, the ability to grow in CDM with one of these amino acids in the form of a peptide is an indication that the peptide is transported. Complications might arise when peptides are (partly) degraded outside the cell or contain the branched-chain amino acids Leu, Ile or Val. In the latter case, a reduced growth rate can be explained in two ways: either the peptide is not efficiently transported, or the expression of the transport system is reduced via the action of CodY (see above). Colonies of an *L. lactis* MG1363 strain containing a transcriptional fusion of the *oppA* promoter with *Escherichia coli lacZ* are, for example, blue on CDM, whereas white on M17, which reflects a 150-fold repression of transcription from P*oppA* when the cells grow in peptide-rich media such as M17 (Guédon *et al*., 2001a,b).

Peptide transport assays in whole *L. lactis* cells are done either by measuring the increase in the internal amino acid pool by reversed-phase high-performance liquid chromatography (Kunji *et al*., 1993) or by using radiolabelled peptides (Detmers *et al*., 1998; Picon *et al*., 2000). *In vivo* uptake experiments, however, are complicated by rapid breakdown of internalized peptides by intracellular peptidases, subsequent efflux of (labelled) amino acids, and binding of peptides to the cell wall (Detmers *et al*., 1998; Picon *et al*., 2000). Accumulation of leu-enkephaline (YGGFL) via Opp, for example, was followed by a rapid efflux of radiolabelled amino acids from the cells (Detmers *et al*., 1998). These complications prevent any detailed kinetic analysis of *in vivo* peptide transport in *L. lactis*. For these reasons, *in vitro* approaches using purified proteins are critical for a comprehensive understanding of all aspects of peptide transport and utilization (Lanfermeijer *et al*., 1999; Detmers *et al*., 2000; Fang *et al*., 2000; Sanz *et al*., 2000; 2003; Doeven *et al*., 2004).

The oligopeptide-binding protein determines the selectivity of the Opp system

To determine the factor(s) that impose peptide uptake selectivity, Charbonnel *et al*. expressed OppA proteins from four distinct *L. lactis* strains in a *L. lactis* MG1363Δ*oppA* strain (Charbonnel *et al*., 2003). Surprisingly, irrespective of the strain from which the OppA protein originated, the peptide utilization patterns were similar to that of the host strain. This suggested that the oligopeptide-binding protein does not play a significant role in determining the transport selectivity of its corresponding ABC uptake system. It was therefore proposed that the membrane complex, OppBCDF, imposes specificity on the transport process. On the contrary, an *in vitro* study, using purified and membrane-reconstituted Opp, clearly showed that OppA determines the uptake selectivity of the Opp ABC transporter (Doeven *et al*., 2004). Binding and transport profiles with combinatorial peptide libraries matched perfectly.

The finding that several of the strains used by Charbonnel *et al*. employed DppP in stead of OppA for the uptake of oligopeptides (Lamarque *et al*., 2004; strains IL1403 and SKM6), or use both Opp and Dpp (strains SK11 and Wg2), clarifies why, in these *in vivo* experiments, OppA was not identified as the main determinant of oligopeptide transport selectivity. The additional transporter (Dpp) transported the oligopeptides. In fact, the observation that an additional peptide-binding protein (DppP) in *L. lactis* IL1403 enables the Dpp system to transport oligopeptides, in addition to di- and tripeptides (as is the case in MG1363), is a further argument that (oligo)peptide-binding proteins determine the uptake selectivity of their cognate ABC transporters.

Competitive inhibition of peptide binding or transport

An indirect way of evaluating binding and/or transport of peptides is to measure competitive inhibition of a reporter peptide, as has been done for the Opp system (Detmers *et al*., 1998; 2000; Helinck *et al*., 2003). Impairment of peptide binding to OppA, leading to reduced transport rates, is the result of competition between the peptide of interest and the reporter peptide for the peptide-binding site of OppA. In one remarkable case however, a tetrapeptide (VGDE) was found that binds to the oligopeptide-binding protein OppA, inhibits transport of the reporter peptide Leu-enkephaline (YGGFL) but, as indicated by growth experiments, is not transported (Charbonnel *et al*., 2003; Helinck *et al*., 2003). *In vitro* binding studies showed that OppA has a low affinity for short peptides and that negatively charged residues reduce the binding affinity even further (Detmers *et al*., 2000). At pH 6.5, which is the common pH of the medium used in growth experiments, the side-chains of Asp ($pK_a = 3.86$) and Glu ($pK_a = 4.25$) are anionic. Under these conditions, *L. lactis* MG1363 does not grow when VGDE is supplied at submillimolar concentrations (Charbonnel *et al*., 2003). However, upon increasing the VGDE concentration to 10 mM or when lowering the pH of the medium to pH 5.0 (and thereby increasing the fraction of protonated species), *L. lactis* was competent in growing on this peptide (unpubl. result). Growth on VGDE requires the *opp* genes and is specifically inhibited by the addition of 100 μM bradykinin (RPPGFSPFR), a cationic peptide that binds to OppA with high affinity ($K_D = 0.1$ μM; Lanfermeijer *et al.* 1999; Detmers *et al*., 2000) and completely blocks *in vitro* peptide uptake via Opp (Doeven *et al*., 2004). These observations further strengthen the notion that any peptide that can bind to OppA is transported via the Opp system.

Length specificity and structure of peptide-binding proteins

A list of peptide-binding proteins characterized to date is given in Table 1. Because of low sequence similarity (usually 20–30% identity) between (oligo)peptide-binding proteins with known peptide-length specificity, it is difficult to predict the specificity of uncharacterized peptide-binding proteins solely on the basis of multiple sequence alignments. Currently, three high-resolution crystal structures of peptide-binding proteins are available: OppA from *Salmonella typhimurium*, DppA from *E. coli* and AppA from *B. subtilis* (Tame *et al*., 1994; Dunten and Mowbray, 1995; Nickitenko *et al*., 1995; Levdikov *et al*., 2004). These proteins bind peptides with 2–5, 2–3 and nine amino acid residues respectively. The structure of AppA with a bound nonapeptide is shown in Fig. 3A. Although the three-dimensional folds of $AppA_{Bs}$, $OppA_{St}$ and $DppA_{Ec}$ are similar, and the peptide-binding sites appear to be located at essentially the same position in each protein, structure-based predictions of peptide-length specificity remain difficult (Levdikov *et al*., 2004). Interestingly, in $AppA_{Bs}$, the

Table 1. (Oligo)peptide-binding proteins with known length specificity.

Organism	Peptide-binding protein	Length of bound peptides	Reference(s)
Escherichia coli[a]	OppA	2–5	Guyer *et al.* (1986)
	DppA	2–3	Dunten and Mowbray (1995) Nickitenko *et al.* (1995)
	MppA	3	Park *et al.* (1998)
Salmonella typhimurium[a]	OppA	2–5	Tame *et al.* (1994)
Xenorhabdus nematophila[a]	OppA1	n.d.	Orchard and Goodrich-Blair (2004)
	OppA2	3	
Enterococcus faecalis	TraC	8	Nakayama *et al.* (1998)
	PrgZ	7	Leonard *et al.* (1996)
Lactococcus lactis MG1363	OppA	4–35	Detmers *et al.* (2000)
	DppA	2–3	Sanz *et al.* (2000)
	DppP	n.d.[b]	Sanz *et al.* (2001)
	OppA2	5[c]	Sanz *et al.* (2004)
Lactococcus lactis IL1403	OppA	n.d.[d]	–
	DppA (OptS)	n.d.[d]	–
	DppP (OptA)	2–9[e]	Sanz *et al.* (2004) Lamarque *et al.* (2004)
Lactobacillus delbrueckii	OppA1	5	Peltoniemi *et al.* (2002)
	OppA2	n.d.	
Streptococcus pneumoniae	AmiA	2–3	Alloing *et al.* (1994)
	AliA	2–7	
	AliB	2–7	
Streptococcus gordonii	HppA	6–7	Jenkinson *et al.* (1996)
	HppG	?	
	HppH	6–7	
Streptococcus thermophilus	AmiA1	6, 11, 22[f]	Garault *et al.* (2002)
	AmiA2	?[f]	
	AmiA3	8, 22, 23[f]	
Streptococcus uberis	OppA1	3–8	Taylor *et al.* (2003)
	OppA2	n.d.	
Streptococcus agalactiae	DppA	2	Samen *et al.* (2004)
	OppA1	2–6	
	OppA2	2–6	
Bacillus subtilis	OppA	3–5	Picon and Van Wely (2001)
	AppA	9[g]	Picon and Van Wely (2001) Levdikov *et al.* (2004)
Borrelia burgdorferi	OppA1	2–7[h]	Lin *et al.* (2001)
	OppA2	2–7[h]	Wang *et al.* (2004)
	OppA3	2–7[h]	
	OppA4	3	
	OppA5	3	

a. In Gram-negative organisms, mostly short peptides (2–5 residues) have been tested because of the inability of longer peptides to pass the outer membrane and reach the transport system.
b. Truncated and therefore not functional.
c. Only one peptide was tested. Probably similar to OppA from *L. lactis* MG1363.
d. Most likely similar to their homologues in *L. lactis* MG1363.
e. Only a few peptides longer than four amino acid residues were tested.
f. The Ami system of *S. thermophilus* transports peptides of 3–23 residues. The length specificities of individual AmiA proteins have not been fully established.
g. A limited number of peptides were tested. Genetic analysis indicated that AppA might bind a range of peptide lengths.
h. Longer peptides might also be bound as binding experiments were done using phage libraries with heptapeptides fused to a phage coat protein.

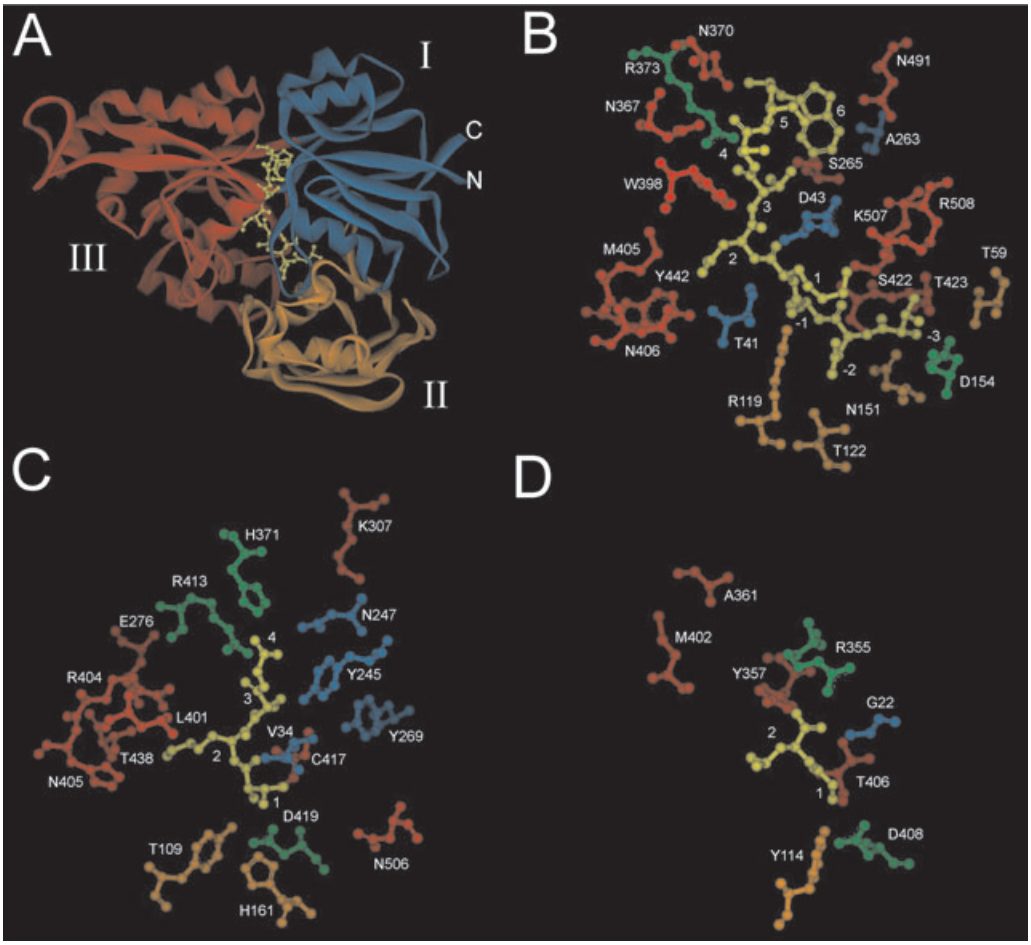

Fig. 3. Structure and binding site architecture of (oligo)peptide-binding proteins. Example of the fold of an (oligo)peptide-binding protein (A). The structure of $AppA_{Bs}$ with a bound nonapeptide (yellow) is shown (Levdikov *et al.*, 2004). Domains I and III engulf the substrate and are common for ABC-type substrate-binding proteins. Domain II is a specific feature of the dipeptide/oligopeptide/nickel transporter family. Architecture of the peptide-binding sites of $AppA_{Bs}$ (B), $OppA_{St}$ (C) and $DppA_{Ec}$ (D). Colour coding is the same as in (A) except for charged protein residues that are involved in salt-bridging either the N- or C-terminus of the bound peptides; these are indicated in green. Residues 4–7 of the nonameric peptide bound to $AppA_{Bs}$ are accommodated in pockets 1–4 (based on the structure of $OppA_{St}$), and the additional pockets are numbered –3, –2, –1, 5 and 6 for easy comparison. Even though the role of domain II in substrate binding and/or transport is not clear, in $AppA_{Bs}$ a few residues contribute to the extended peptide-binding site (B).

location of positions 4–7 of a bound nonameric peptide corresponds to the positions 1–4 in an $OppA_{St}$ substrate (Fig. 3B and C). Similarly, positions 1 and 2 of the dipeptide bound in $DppA_{Ec}$ superimpose on positions 4 and 5 in the $AppA_{Bs}$-bound peptide (Fig. 3B and D). Residues 1–3 of the ligand bound to $AppA_{Bs}$ are accommodated in an enlarged binding pocket because of a 3-residue shorter loop (residues 421–427) and displacement of an α-helix (151–156).

Alignment of the three (oligo)peptide-binding proteins of known structure with the well-characterized lactococcal (oligo)peptide-binding proteins OppA, DppA and DppP, and other peptide-binding proteins listed in Table 1 indicates that, in general, residues contributing to the peptide-binding site are poorly conserved (not shown; for alignments of peptide-binding proteins mentioned here or elsewhere, see Detmers *et al.*, 2001 and Levdikov *et al.*, 2004). A notable exception is Asp^{419} of $OppA_{St}$ (the equivalent of Asp^{408} of $DppA_{Ec}$), which forms a salt-bridge with the N-terminal amino-group of the peptide in the crystal structures (Fig. 3C and D). Although not present in $OppA_{Ll}$ and $AppA_{Bs}$, this residue is conserved in many other characterized peptide-binding proteins (Table 1) as well as in other (putative) (oligo)peptide-binding proteins (Detmers *et al.*, 2001). This suggests that the 'anchoring' of peptides via their N-terminal α-amino groups is similar in most (oligo)peptide-binding proteins. Furthermore, substrate-binding proteins that do not bind peptides but are homologous to (oligo)peptide-binding proteins, for example, the haem-binding protein HbpA from *Haemophilus influen-*

zae, the agrocinopines-binding protein AccA from *Agrobacterium tumefaciens*, and the nickel-binding protein NikA from *E. coli*, do not contain the conserved aspartate, which thus seems to be specific for peptide-binding proteins (Detmers *et al*., 2001). The residue in $AppA_{Bs}$ that is at the equivalent position of Asp^{419} in $OppA_{St}$ is Ser^{422}, which is involved in hydrogen bonding with the N-terminus of the peptide (Fig. 3B). This serine is also present in $OppA_{Ll}$ which might indicate that binding of peptide N-termini in $AppA_{Bs}$ and $OppA_{Ll}$ is somewhat different from that in other (oligo)peptide-binding proteins. Asp^{154}, which forms a salt-bridge with the N-terminal residue of the nonameric peptide bound to $AppA_{Bs}$ (Fig. 3B), is not present in $OppA_{Ll}$.

Charged residues that were found to form salt-bridges with C-terminal residues of the bound peptide (like Arg^{373} in $AppA_{Bs}$, Arg^{413} and His^{371} in $OppA_{St}$, and Arg^{355} in $DppA_{Ec}$; Fig. 3B–D), are often absent in other (oligo)peptide-binding proteins (Detmers *et al*., 2001; Levdikov *et al*., 2004). Despite these possible differences in the interactions of the proteins with the N- and C-termini of the peptides, the majority of the protein–peptide contacts are formed via the backbone C=O and N-H groups, which facilitates sequence-independent binding of peptides. This phenomenon occurs similarly in each of the peptide-binding proteins for which a structure is available. Interactions with the side-chains of the peptide would impose a high level of specificity toward peptides containing specific amino acid residues. The fact that the peptide–protein interactions are preferably of a non-specific nature might explain why the peptide-binding sites are so poorly conserved and predictions of length specificity of (putative) (oligo)peptide-binding proteins are so difficult. It would be important to know the binding mechanisms of proteins that are thought to be highly specific for certain peptides, such as the pheromone-binding proteins TraC and PrgZ of *Enterococcus faecalis* (Leonard *et al*., 1996; Nakayama *et al*., 1998; Clewell *et al*., 2000). However, detailed peptide binding studies are not available for any of these proteins.

Phylogenetic analyses of characterized (oligo)peptide-binding proteins indicate that binding proteins belonging to different transport systems can be clearly distinguished from each other (Fig. 4). The lactococcal Dpp-binding proteins cluster together, and appear distantly related to the OppA proteins from *Streptococcus agalactiae*, *Strepto-*

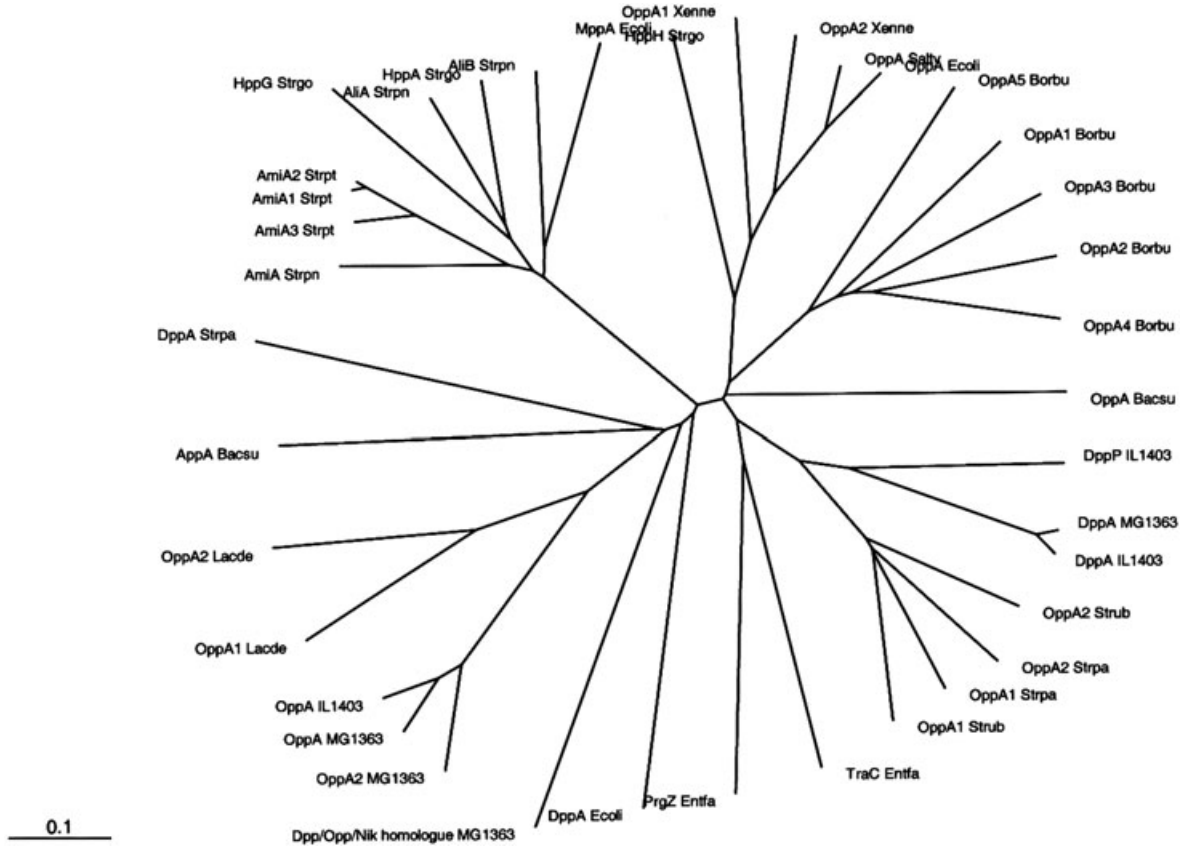

Fig. 4. Phylogenetic tree of (oligo)peptide-binding proteins with known length specificity. Bacsu, *B. subtilis*; Borbu, *B. burgdorferi*; Ecoli, *E. coli*; Entfa, *E. faecalis*; IL1403, *L. lactis* IL1403; Lacde, *L. delbrueckii*; MG1363, *L. lactis* MG1363; Salty, *S. typhymurium*; Strgo, *S. gordonii*; Strpa, *S. agalactiae*; Strpn, *S. pneumoniae*; Strpt, *S. thermophilus*; Strub, *S. uberis*; Xenne, *X. nematophila*.

coccus uberis, and the pheromone-binding proteins TraC and PrgZ from *E. faecalis*. The lactococcal OppAs (including OppA2 and the novel dipeptide/oligopeptide/nickel-binding protein from MG1363), together with those from *Lactobacillus delbrueckii*, form a loose cluster with $AppA_{Bs}$, $DppA_{Sa}$ and $DppA_{Ec}$ but, overall, the identity between the proteins is low. The remaining streptococcal proteins (Ami and Ali from *Streptococcus thermophilus* and *Streptococcus pneumoniae*, and Hpp from *Streptococcus gordonii*) all fall in one large group. The borrelial OppAs seem closely related to OppA and MppA from *E. coli,* and OppA from *Streptococcus typhymurium*, as do OppA1 and OppA2 from *Xenorhabdus nematophila*. Clearly, proteins with different peptide-length specificities cluster together in the phylogenetic tree, again exemplifying the non-conservative nature of the peptide-binding sites. The most striking example is probably $AmiA_{Sp}$, which, although it binds only di- and tripeptides, clusters with other Ami/Ali proteins, that bind peptides of up to and including 23 amino acids. Generally (oligo)peptide-binding proteins using the same membrane complex cluster closely together. This is emphasized by the observation that OppAs from *Borrelia burgdorferi* could complement an *E. coli oppA* deletion strain by functionally interacting with the *E. coli* OppBCDF complex (Lin *et al*., 2001) and by the fact that $OppA2_{Ll}$ could restore function to the Opp permease in a MG1363Δ*oppA* strain (Sanz *et al*., 2004).

Multiple sequence alignments of all the (oligo)peptide-binding proteins listed in Table 1 reveal that the lactococcal OppAs and streptococcal Ami and Ali have several extensions compared to the other peptide-binding proteins (not shown). These particular sites in the proteins could be involved in the specific recognition by their cognate membrane complexes or be involved in the binding of extremely long peptides (up to and including 23 or 35 amino acid residues for the Ami- or Opp-binding proteins respectively), which has been reported only for these two systems.

Conclusions

An overview of peptide transport in *L. lactis* species has been presented, and the implications of recent findings for previously proposed hypotheses were discussed. *L. lactis* MG1363 uses DtpT and Dpp for the uptake of short peptides (2–3 residues) and Opp for the uptake of oligopeptides. Some other lactococcal strains also use the Dpp system for oligopeptide uptake. Dpp appears to be a versatile peptide transporter that uses multiple peptide-binding proteins (DppA and DppP), at least in *L. lactis* IL1403, SK11, SKM6 and Wg2. This situation is similar to that of streptococcal and borrelial peptide uptake systems (Ami/Ali/Opp), which use multiple peptide-binding proteins with overlapping specificities (Alloing *et al*., 1994; Garault *et al*., 2002; Samen *et al*., 2004; Wang *et al*., 2004). Contrary to some recent reports (Charbonnel *et al*., 2003; Helinck *et al*., 2003), we provide further evidence for the notion that the selectivity of the peptide ABC transporters is imposed by the specificity of the (oligo)peptide-binding proteins and that the membrane-embedded translocator complexes are non-selective.

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